NREL-Amoco CRADA Phase 3

Bench Scale Report 2.2

Effect of Cell Mass Spiking on the Continuous Fermentation of Corn Fiber Hydrolyzate by L1400 (pLNH33)

Project Title: Amoco-NREL CRADA (Phase 3)
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Objective

To determine the effect of adding fresh cells and increasing the cell concentration on the cofermentation of glucose and xylose during the continuous steady state fermentation of corn fiber hydrolyzate. The spiking experiment was performed on a continuous corn fiber hydrolyzate fermentation already in progress (see report 2.1).

.Materials and Methods

Yeast strain

The organism used in these studies was the genetically engineered recombinant yeast L1400 (pLNH33). The seed vials were prepared by growing the cells in YEPX media for 18 hours, diluting 1/2 with a 40% (w/v) glycerol solution. and finally freezing them quickly. The organism originated at Purdue University (Dr. Nancy Ho) and was supplied to NREL by Amoco Corporation.

Inoculum Preparation

Two separate step additions (spikes) were carried out: the first at a 10% (v/v) inoculum level and the second at a 50% (v/v) inoculum level. The terms "10% (v/v) level" and "50% (v/v) level" describe the volume of the prepared fresh inoculum (as a percentage of the reactor volume) to be added to the fermentor. The following protocol was used to prepare the two inocula. A 1-mL frozen vial stored at -70" C was thawed at mom temperature and inoculated into 50 mL YEPX with 2% (w/w) xylose and 1%(w/w) CSL media. The first stage inoculum was incubated at 30" C in a rotary shaker (150 rpm) for 30 hours. The second stage inoculum was started with a 10% (v/v) seed from the first stage added to 90 mL media. The media composition was the same as for the first stage, and the second inoculation lasted for 18 hours.

Growth Media

The corn fiber medium was prepared by adding 2% (w/w) corn steep liquor and 1%(w/w) yeast extract to 15 liters of pretreated corn fiber supernatant. The corn fiber was pretreated using the proprietary (APR) Amoco process. The hydrolyzate (pH 1.6) was autoclaved at 121" C for 90 minutes.

Inoculation

The 10% inoculum spiking was performed by replacing 100 mL of fermentation medium by 100 mL of fresh inoculum at the time indicated by the first arrow in **Figure 1.** This increased cell concentration in the fermentor by four times. The 50% inoculum had a starting volume of 500 mL and was centrifuged down to a final volume of 50 mL to concentrate the cells. The concentrated inoculum was added to the fermentor after removing 50 mL of media from the vessel. This increased the cell concentration by 25 times to 2.3×10^8 CFUs.

Growth Conditions

The continuous fermentation temperature was maintained at 30" C, the pH at *5.00,* and the agitation speed at 150 rpm. The steady state volume was 1 liter with a resident time of 72 hours.

Analytical techniques

Glucose and ethanol concentrations were determined using a Hewlett Packard 1090 HPLC equipped with a 1047 IR detector, HPX-87XH, and a HPX-87XP column. Column temperature was *85"* C. Samples were centrifuged and sterile filtered (0.2μ).

Results and Discussion

As seen in Figure 1, the glucose and xylose levels remained unchanged after the 10% inoculation, and ethanol production was not affected. In contrast, the glucose concentration dropped by about 1 g/L for 25 hours after the 50% inoculation and then returned to the pre-inoculation steady state level. The xylose concentration dropped by about *5* g/L to 30 g/L and slowly returned to the pre-inoculation steady state level after one residence time. The ethanol concentration increased immediately following the 50% inoculation from 15 g/L to 19 g/L and gradually dropped to 16 g/L. This demonstrates a significant improvement in ethanol for the short term and a small improvement (1 to 2 g/L) in the long term (steady state concentration).

The ethanol metabolic yield (% theoretical) went down from 88.9% (574 hours) to 82.6% (644 hours), but the productivity improved from 0.201 to 0.264 g/L·h, which would result in 1.5 g/L more ethanol being made each day. It must be noted that these comparisons are between the steady state prior to inoculation (574 hours) and the period right after the spiking (644 hours), when xylose consumption and ethanol production are at their *peak.*

The effect of inoculum addition on the oligomeric to monomeric sugar ratio during the course of the experiment (Figures 2-6) is rather inconclusive. Glucose seems to be "moving" toward the monomeric form during the fermentation, whereas the oligomeric xylose, galactose, and arabinose concentrations seem to remain the same. Unfortunately, the measured total glucose concentration in the feed (monomeric and oligomeric) vaned, although experimentally it was kept constant. As a result, it is believed that analytical inaccuracies introduce uncertainty into the oligomer/monomer analysis.

The carbon balances were calculated for four separate time points (see spreadsheets 1-4). The first carbon balance was performed on sample T20 taken at 354 hours (before the 10% inoculum spiking). The second sample, T31, was taken at 574 hours and represented the steady state conditions just before the 50% inoculation. The third sample, T37, was taken at 644 hours, just after the 50% inoculation and at a time when the highest rates of xylose and glucose consumption

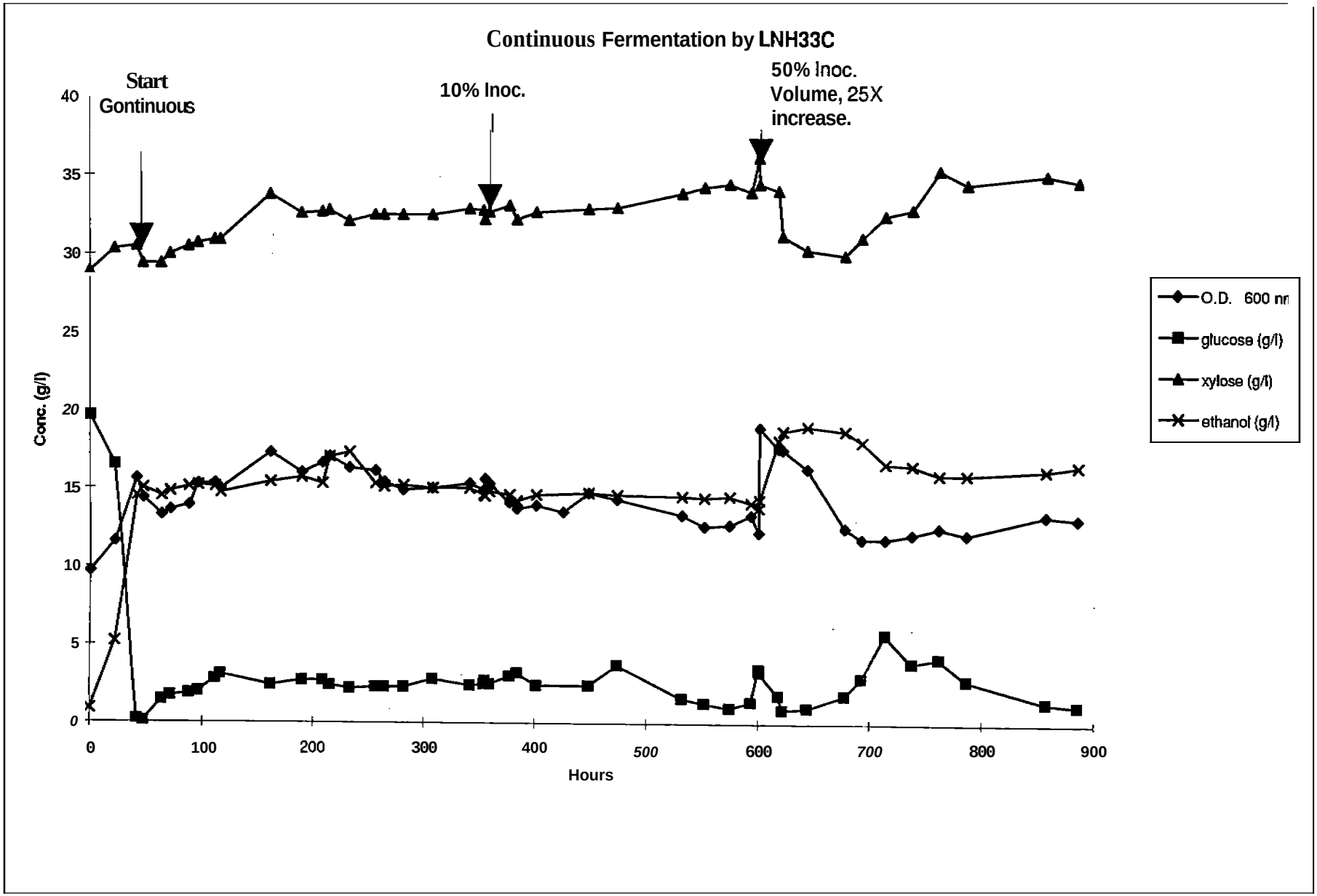
and ethanol production (19 g/L) were observed. The fourth sample was T45; it was taken at 885 hours at the end of the continuous fermentation. Overall, the carbon balance closures were satisfactory, ranging between 92% and 104%.

Interestingly, the product and ethanol yields for sample T20 were 30 to 40% lower than calculated for later time points, respectively. This appears to be related to the difficulty in sugar oligomers, as mentioned earlier. The product yields for the remaining time points approached 100%. The ethanol metabolic yields for T31 and T37 were just above 80% and the T45 sample was over 100%.

Conclusions

Increasing the cell concentration with the 50% inoculum spike improved xylose utilization and ethanol production, but only to a small extent at the steady state level. These increases indicate that plasmid may have been lost during the early batch operation, when glucose levels were high and there was little selection pressure on the yeast to maintain its plasmid-borne xylose genes. However, the improvement is small, and a continuous inoculum may not significantly improve the continuous fermentation. Nevertheless, the concept of continuous inoculation should be tested experimentally, because, in addition to the fresh inocdum, cell adaptation may also play a positive role in enhancing the performance of hydrolyzate fermentation.

Graph 1


Continuous Fermentation by LNH33C
Start Gontinuous
10% Inoc.
50% Inoc. Volume, 25X increase.
Conc. (g/l)
Hours
O.D. 600 nr
glucose (g/l)
xylose (g/l)
ethanol (g/l)

SSF CARBON BALANCE: Liquid Fraction of Corn Fiber

T20: **354 hours**

Overall C6-Sugar Conversion:	44.4%
Overall C5-Sugar Conversion:	2.2%
Ethanol Metabolic Yield (% theor):	88.9%
Productivity (g/l h):	0.201
Total Carbon Recovery:	100%

T31: 574 hours

Overall C6-Sugar Conversion:	44.4%
Overall C5-Sugar Conversion:	2.2%
Ethanol Metabolic Yield (% theor):	88.9%
Productivity (g/l h):	0.201
Total Carbon Recovery:	100%

T37: 644 hours

Overall C6-Sugar Conversion:	58.6%
Overall C5-Sugar Conversion:	9.8%
Ethanol Metabolic Yield (% theor):	82.6%
Productivity (g/l h):	0.264
Total Carbon Recovery:	99%

T45: 885 hours

Overall C6-Sugar Conversion:	58.6%
Overall C5-Sugar Conversion:	9.8%
Ethanol Metabolic Yield (% theor):	82.6%
Productivity (g/l h):	0.264
Total Carbon Recovery:	99%

SSF CARBON BALANCE: Liquid Fraction of Corn Fiber T20: 354 hours

Run: Continuous Fermentation of Hydrolysate Liquor by LNH33C
Pretreatment:

)LIDS BALANCE	In	Out
Lignin (%):	0	0.00
uble Solids (%):	0.00	0.00

Cellulose Conversion:	#DIV/0!
Overall C6-Sugar Conversion:	57.3%
Overall C5-Sugar Conversion:	11.2%
Ethanol Process Yield (% theor):	18.8%
Ethanol Metabolic Yield (% theor):	58.3%

Carbon Balance: SSF

	Carbon In						Carbon Out						Conversion	Yield	Yield
	In Solids			In Liquor		Total	In Solids			in Liquor		Total In-Out)/In	g product/	product/	
Component	% dry wt)	(C-mole	otal In)	(g/L)	(C-mole	otal In)	(C-mole/l	% dry wt)	(C-mole	al Out)	(g/L)	(C-mole	al Out)	(C-mole	(%)	100 g C6 c	100 g C6+C5 cc
Cellobiose				0.00	0.000	0.000					0.00	0.000	0.000				
Glucose	0	0.000	0.0	55.30	1.842 100.0	1.842	0.00	0.000	0.0	18.60	0.626 100.0	0.626	66.00				
Galactose	0	0.000	0.0	14.00	0.466 100.0	0.466	0.00	0.000	0.0	10.80	0.360 100.0	0.360	22.86				
Mannose	0	0.000	#####	0.00	0.000 #####	0.000	0.00	0.000	#P###	0.00	0.000#####	0.000	#DIV/0!				
Xylose	0	0.000	0.0	44.40	1.479 100.0	1.479	0.00	0.000	0.0	44.40	1.479 100.0	1.479	0.00				
Arabinose	0	0.000	0.0	38.30	1.276 100.0	1.276	0.00	0.000	0.0	29.00	0.966 100.0	0.966	24.28				
Lignin	0	0.000	#####	0.00	0.000 #####	0.000	0.00	0.000	#####	0.00	0.000 #####	0.000	#DIV/0!				
	0																
Ethanol				0.00	0.000	0.000				14.60	0.634	0.634		36.78	29.80		
Cell Mass				0.00	0.000	0.000				4.90	0.196	0.196		12.34	10.00		
Carbon Dioxide				0.00						0.00	0.317	0.317		35.13	28.66		
Glycerol				0.20	0.007	0.007				1.40	0.046	0.046		3.02	2.45		
Acetic Acid				6.60	0.220	0.220				6.60	0.220	0.220		0.00	0.00		
Lactic Acid				4.00	0.133	0.133				4.20	0.140	0,140		0.50	0.41		
Succinic Acid				0.00	0.000	0.000				0.00	0.000	0.000		0.00	0.00		
Total	0	0.000	0.0		5.422 100.0	5.422	0.00	0.000	0.0		4.982 100.0	4.982		87.78	71.12		

C - RECOVERY 91.9%

'SSF CARBON BALANCE: Liquid Fraction of Corn Fiber T31: 574 hours

Run: Continuous Fermentation of Hydrolysate Liquor by LNH33C
Pretreatment:

)LIDS BALANCE	In	Out
Lignin (%):	0	0.00
uble Solids (%):	0.00	0.00

Cellulose Conversion:	#DIV/0!
Overall C6-Sugar Conversion:	44.4%
Overall C5-Sugar Conversion:	2.2%
Ethanol Process Yield (% theor):	17.7%
Ethanol Metabolic Yield (% theor):	88.9%

Carbon Balance: SSF

	Carbon In						Carbon Out						Conversion		Yield	Yield
	In Solids			In Liquor		Total	In Solids			In Liquor			Total (In-Out)/In		g product/	product/
Component	% dry wt)	(C-mole	otal In)	(g/L)	(C-mole	otal In) (C-mole/L	% dry wt)	(C-mole	al Out)	(g/L)	(C-mole	al Out)	(C-mole	(%)	100 g C6 c	100 g C6+C5 cc
Cellobiose				0.00	0.000		0.000				0.00	0.000		0.000		
Glucose	0	0.000	0.0	49.70	1,655	100.0	1.655	0.00	0.000	0.0	25.50	0.849	100.0	0.849	48.69	
Galactose	0	0.000	0.0	12.60	0.420	100.0	0.420	0.00	0.000	0.0	12.00	0.400	100.0	0.400	4.76	
Mannose	0	0.000	0.0	5.10	0.170	100.0	0.170	0.00	0.000	#####	0.00	0.000	#####	0.000	100.00	
Xylose	0	0.000	0.0	60.40	2.012	100.0	2.012	0.00	0.000	0.0	58.90	1.902	100.0	1,962	2.40	
Arabinose	0	0.000	0.0	32.60	1.086	100.0	1.086	0.00	0.000	0.0	32.10	1.069	100.0	1,069	1.53	
Lignin	0	0.000	#####	0.00	0.000	#####	0.000	0.00	0.000	#####	0.00	0.000	#####	0.000	#DIV/0!	
	0															
Ethanol				0.00	0.000		0.000				14.50	0.629		0.629		48.49	45.45
Cell Mass				0.00	0.000		0.000				2.70	0.108		0.108		9.03	8.46
Carbon Dioxide				0.00								0.315		0.315		46.33	43.42
Glycerol				0.20	0,007		0,007				1.10	0.036		0.036		3.01	2.82
Acetic Acid				6.60	0.220		0.220				6.60	0.220		0.220		0.00	0.00
Lactic Acid				4.00	0.133		0.133				4.20	0.140		0.140		0.67	0.63
Succinic Acid				0.00	0.000		0.000				0.00	0.000		0.000		0.00	0.00
Total	0	0.000	0.0		5.702	100.0	5.702	0.00	0.000	0.0		5.727	100.0	5.727		107.53	100.79

C - RECOVERY: 100.4%

SSF CARBON BALANCE: Liquid Fraction of Corn Fiber **T37: 644 hours**

Run: **Continuous Fermentation of Hydrolysate Liquor by LNH33C**
Pretreatment:

)LIDS BALANCE	*In*	*Out*
Lignin (%):	0	0.00
uMe Solids (%):	***0.00***	0.00

Cellulose Conversion:	#DIV/0!
Overall C6-Sugar Conversion:	58.6%
Overall C5-Sugar Conversion:	9.8%
Ethanol Process Yield (% theor):	24.0%
Ethanol Metabolic Yield (% theor):	82.6%

Carbon Balance: SSF

	Carbon In							Carbon Out						Conversion	Yield	Yield
	In Solids			*In Liquor*			Total	*In Solids*			*In Liquor*			*Total In-Out)/In*	g product/	product/
Component	*6 dry wt)*	(C-mole	otal In)	(g/L)	(C-mole	otal In)	(C-mole/L	% dry wt)	(C-mole	al Out)	(g/L)	(C-mole	al Out)	(C-mole	(%)	100 g C6 c	100 g C6+C5 cc
Cellobiose				0.00	0.000		***0.000***				***0.00***	0.000		0.000			
Glucose	***0***	0.000	***0.0***	47.00	1.565	*100.0*	1.565	0.00	0.000	0.0	15.00	0.500	*100.0*	**0.500**	68.09		
Galactose	***0***	0.000	***0.0***	11.20	0.373	*100.0*	0.373	0.00	0.000	0.0	10.30	0.343	*100.0*	0.343	8.04		
Mannose	***0***	0.000	*0.0*	***2.90***	**0.097**	*100.0*	0.097	0.00	0.000	***#####***	***0.00***	***0.000#####***		0.000	100.00		
Xylose	***0***	0.000	*0.0*	**58.30**	1.942	*100.0*	1.942	0.00	0.000	0.0	50.70	1.689	*100.0*	1.689	13.04		
Arabinose	0	0.000	*0.0*	35.50	1.182	*100.0*	1.182	0.00	0.000	*0.0*	**33.90**	1.129	*100.0*	1.129	4.51		
Lignin	0.0	0.000	***#####***	0.00	***0.000#####***		***0.000***	***0.00***	0.000	***#####***	**0.00**	0.000	***#####***	0.000	#DIV/0!		
Ethanol				*0.00*	***0.000***		***0.000***				19.00	0.825		**0.825**		**53.07**	42.22
Cell Mass				*0.00*	**0.000**		***0.000***				4.17	0.166		0.166		11.65	9.27
Carbon Dioxide				***0.00***								0.412		0.412		50.70	40.33
Glycerol				**0.20**	***0.007***		***0.007***				2.30	0.075		0.075		**5.87**	4.67
Acetic Acid				6.60	**0.220**		**0.220**				6.40	0.213		0.213		-0.56	-0.44
Lactic Acid				**4.00**	**0.133**		**0.133**				4.10	0.137		0.137		0.28	0.22
Succinic Acid				*0.00*	0.000		0.000				0.00	0.000		0.000		0.00	***0.00***
Total	*0*	0.000	***0.0***		5.518	*100.0*	***5.518***	*0.00*	*0.000*	***0.0***		5.488	*100.0*	***5.488***		***123.01***	***96.27***

C - RECOVERY: **99.46%**

SSF CARBON BALANCE: Liquid Fraction of Corn Fiber T45: 885 hours

Run, Continuous Fermentation of Hydrolysate Liquor by LNH33C

Pretreatment:

)LIDS BALANCE	*In*	*Out*
Ltgnln(%):	0	0.00
uble Solids (%):	0 00	0.00

Cellulose Conversion:	#DIV/0!
Overall C6-Sugar Conversion:	52.7%
Overall C5-Sugar Conversion:	4.1%
Ethanol Process Yield (% theor)	24.3%
Ethanol Metabolic Yield (% theor)	101.8%

Carbon *Balance: SSF*

	Carbon In							Carbon Out						Convenlon	Yield	Yield
	In *Solids*			*In Liquor*			***Total***	***In Solids***			In *Liquor*			*Total*		
Component	(g dry wt)	(C-moleotal In)		(g/L)	(C-moleotat In)		(C-mole/l	(g dry wt)	(C-moleal Out)		(g/L)	(C-moleal Out)		(C-mole	(%)	*(Total In-Out)/In g* product/ 100 *g* C6 c	product/ 100 g C6+C5 cc
Cellobiose				0.00	0.000		*0* 000				**0.00**	0.000		**0.000**			
Glucose	**0**	0.000	*0.0*	**44 20**	**1472**	*100.0*	**1472**	0.00	0.000	**0.0**	15.10	0.503	*100.0*	0.503	**65.84**		
Galactose	0	0.000	*0.0*	**990**	**0330**	*100.0*	**0330**	**000**	0.000	**0.0**	10.50	*0,350*	*100.0*	0.350	**-6.06**		
Mannose	0	0.000	***#####***	0.00	0.000	***#####***	***0000***	***0 00***	**0.000**	***#####***	**0.00**	0.000	***#####***	0.000	#DIV/0!		
Xylose	0	**0000**	*0.0*	**4760**	**1585**	*100.0*	1.585	0.00	0.000	*0.0*	45.90	**1.529**	***100.0***	**1.529**	**3.57**		
Arabinose	0	0.000	*0.0*	31 **30**	**1042**	*100.0*	**1042**	0.00	0.000	**0.0**	29.80	*0.992*	*100.0*	0.992	**4.79**		
Lignin	0 0	0.000	***#####***	*000*	0.000	***#####***	0.000	0.00	0.000	***#####***	*0.00*	0.000	***#####***	0.000	#DIV/0!		
Ethanol				*000*	0.000		***0 000***				**16.50**	**0.716**		**0.716**		***57.89***	52.05
Cell Mass				*000*	0.000		0.000				**3.20**	**0.128**		**0.128**		**11.23**	10.09
Carbon Dioxide				0 **00**							0.00	**0.358**		**0.358**		55.31	49.72
Glycerol				**022**	**0007**		**0**007				**1.20**	**0.039**		**0.039**		**3.44**	3.09
Acetic Acid				6.50	**0216**		**0.216**				6.60	**0.220**		**0.220**		**0.35**	**0.32**
Lactic Acid				**414**	**0138**		**0.130**				**4.20**	0.140		**0.140**		**0.21**	**0.19**
Succinic Acid				0.00	0.000		0.000				0.00	0.000		0.000		**0.00**	0.00
Total	*0*	0.000	*0.0*		**4,791**	*100.0*	***4.791***	*0.00*	*0.000*	**0.0**		**4.975**	*100.0*	**4.975**		***129.43***	***175.46***

C - RECOVERY **103.8%**

Graph 2 (T20, **354 hours)**

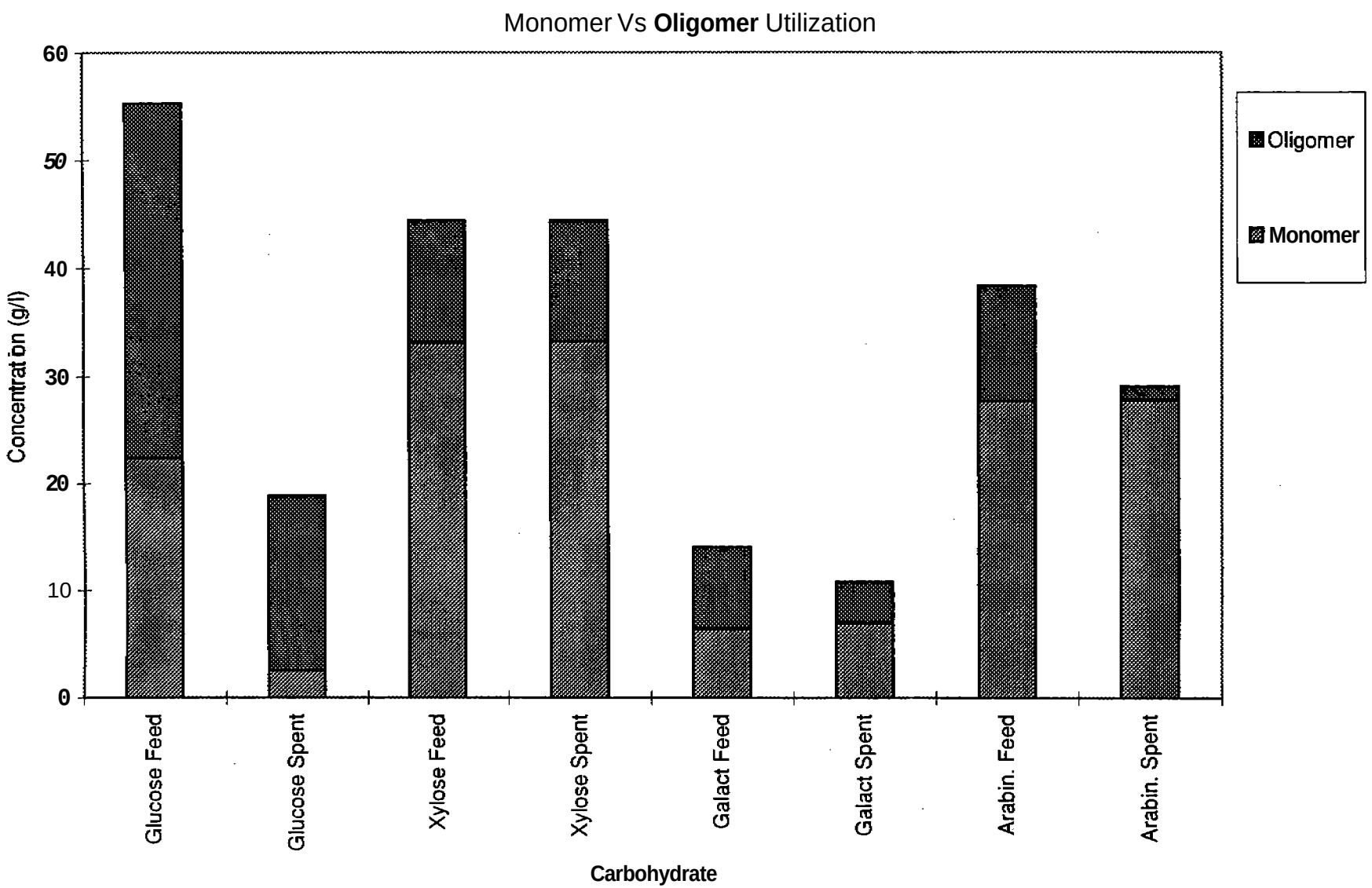
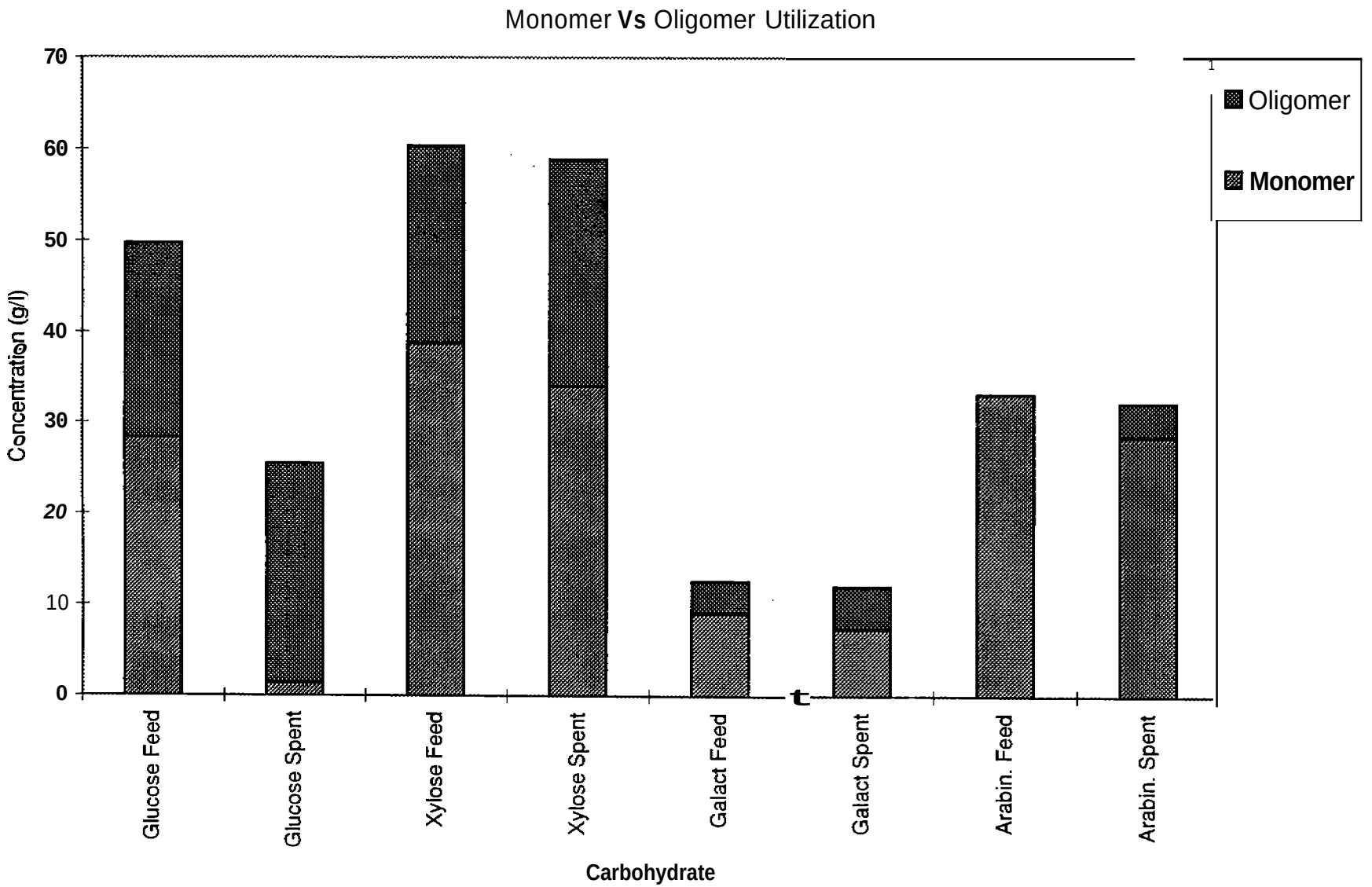
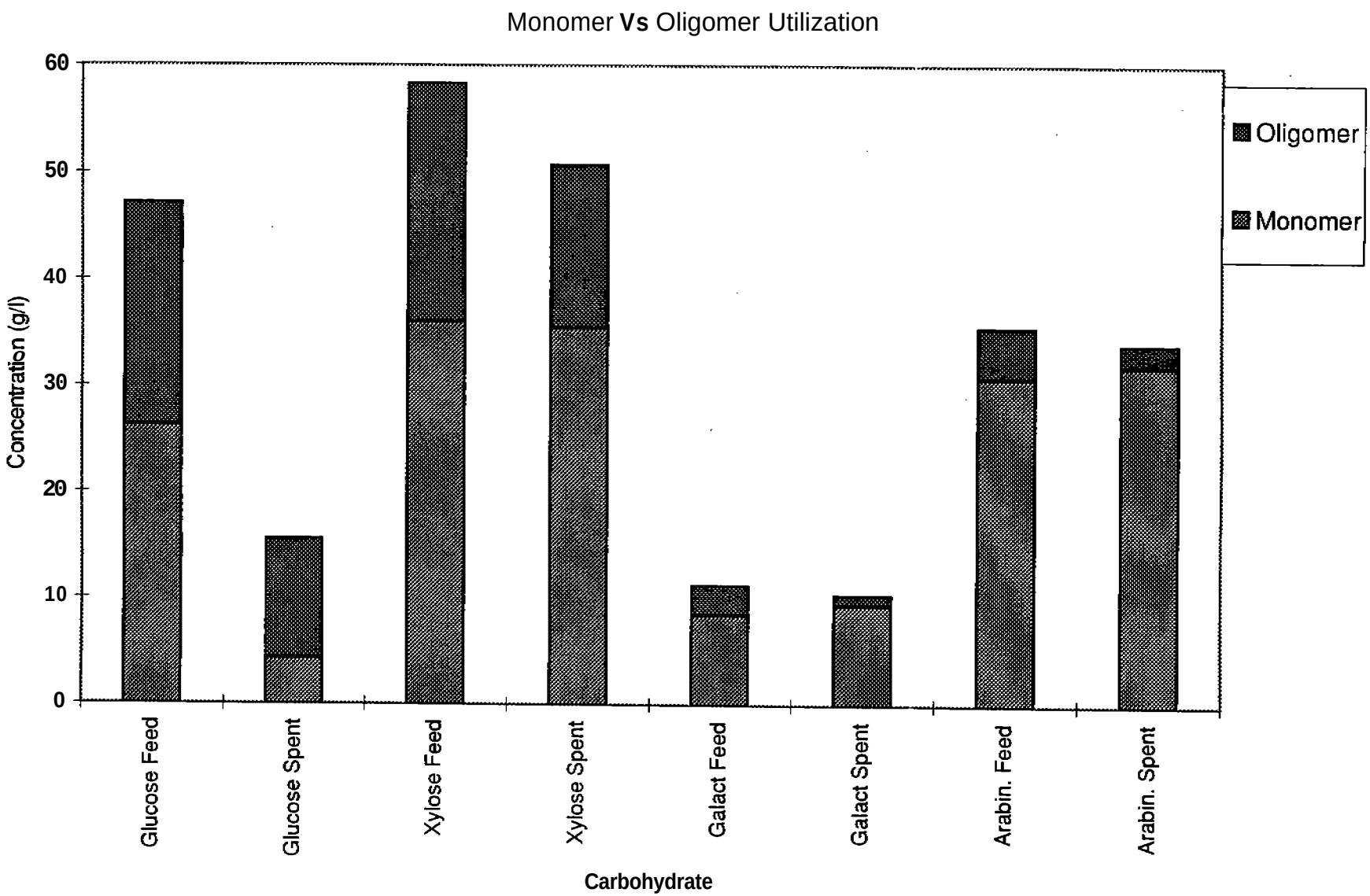


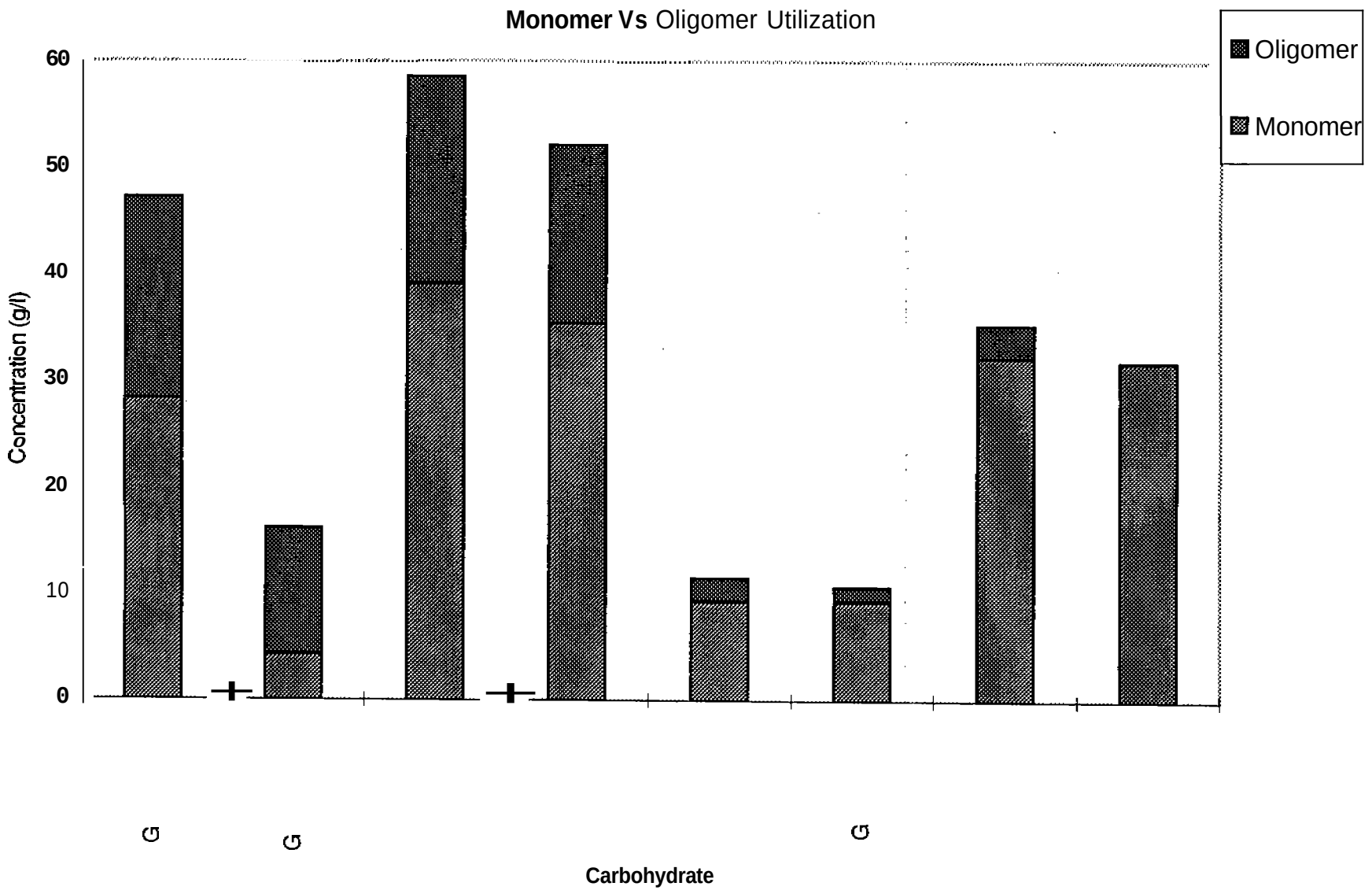
Chart 3 (T32, 594 hours)



Graph 4 (T40, 714 hours)



Graph 5 (T42, 762 hours)



Graph **6** (T45, 886 hours)